

Expression of the thymus leukemia antigen in mouse intestinal epithelium

(major histocompatibility complex/class Ib molecules/mucosal immunology)

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ABSTRACT The *Qa* and *Tla* regions of the mouse major histocompatibility complex contain a series of genes encoding proteins with structural similarity to the class I transplantation antigens of the same complex. In contrast to the genes encoding the transplantation antigens, the *Qa* and *Tla* genes show very little polymorphism. Function(s) of the proteins encoded by the *Qa* and *Tla* loci remain an enigma. Recently, the protein products of the *Qa* and *Tla* loci, often referred to as class Ib major histocompatibility complex molecules, have been proposed to present antigen to $\gamma\delta$ T cells. In mice, $\gamma\delta$ T cells have been found concentrated in several epithelial barriers and in the skin; yet, expression of serologically detectable Tla antigens is believed restricted to thymocytes, activated T lymphocytes, and some T-cell leukemias. Here we report that luminal epithelial cells of the mouse small intestine express the thymus leukemia antigen (TLA). We also find that, unlike T cells in Peyer's patches, a significant fraction of intestinal epithelial lymphocytes also express TLA. RNA prepared from intestinal cells contains transcripts of the *T18^d* gene, which encodes TLA. These data extend the known expression profile of TLA molecules to mature lymphocytes and to nonhematopoietic cells. These data also demonstrate the specific expression of TLA on antigen-presenting cells in a site enriched for T cells that express $\gamma\delta$ T-cell antigen receptor.

The major histocompatibility complex (MHC) of the mouse spans over 600 kilobases (kb) on chromosome 17 and contains the genes encoding both class I and class II MHC molecules (for review, see ref. 1). The genes of the class I family can be subdivided into the *K*, *D*, *Qa*, *Tla*, and *Hmt* regions based on immunogenetic mapping of the MHC locus. Function of the classical transplantation antigens of the *K* and *D* regions of the MHC in presentation of peptide antigens to cytotoxic T lymphocytes is well established. However, function(s) of the more telomeric genes of the *Qa*, *Tla*, and *Hmt* regions of the MHC, sometimes collectively referred to as class Ib genes, remain unknown (for review, see ref. 2).

Whereas the surface expression of the *K* and *D* transplantation antigens is seen on most somatic tissues, the expression of class Ib molecules is believed to be more restricted. For example, expression of the thymus leukemia antigen (TLA), a serological determinant encoded by the *Tla* region, has been observed primarily in thymocytes, activated T lymphocytes, and in some T-cell leukemias (3, 4). Moreover, compared with classical transplantation genes of the *H-2* region, which display a high degree of polymorphism between individuals, genes of the *Tla* region are less polymorphic (5). The TLA protein is closely related structurally to the *K* and *D* transplantation antigens and associates with β_2 -

microglobulin. Yet, the expression profile described for TLA and its lack of polymorphism are not expected for a molecule with a role in antigen presentation.

Although cell-mediated immune responses to the TLA molecule have not been reported, recently several groups have described $\gamma\delta$ T-cell lines and T-cell hybridomas reactive to allogeneic *Tla*-encoded molecules or that are specific for antigen in the context of a *Tla* gene product (6-8). These findings have led to the hypothesis that one function of the products of the *Tla* region may be to present antigens, possibly conserved antigens or "stress" proteins, to T cells that express $\gamma\delta$ T-cell antigen receptor (TCR) (9, 10). If this hypothesis were correct, *Tla* region genes should be expressed on antigen-presenting cells in a site (or sites) that contain $\gamma\delta$ T cells. One such site in the mouse is the intestinal epithelium, where a significant fraction of T cells express $\gamma\delta$ TCR. Indeed, Itohara *et al.* (11) have recently shown the presence of $\gamma\delta$ T cells in several mucosal epithelial barriers. Here we report that TLA, a serological determinant encoded by the *Tla* locus of the MHC, is expressed on epithelial cells of the mouse small intestine but not in other anatomical sites containing $\gamma\delta$ T cells. We also demonstrate that the *T18^d* gene (formerly referred to as the *T13^c* gene), which encodes TLA is expressed in the intestinal epithelium. Moreover, we show that, in contrast to other T lymphocytes in the intestine, a significant fraction of intestinal lymphocytes (IEL) also express TLA.

MATERIALS AND METHODS

Mice. B10.BR, BALB/c, and C57BL/6 mice were bred in our animal colony from breeding pairs originally obtained from The Jackson Laboratory. Eight- to 12-week-old mice of both sexes were used in these studies. BALB/c *scid/scid* mice were a gift of O. Witte (University of California at Los Angeles).

Preparation of Cells. Thymuses were dissected into RPMI 1640 medium, and single-cell suspensions were prepared by dissociating the tissue with glass microscope slides. The cells were passed through nylon mesh and maintained on ice in RPMI 1640/10% fetal bovine serum until use. IEL were prepared essentially as described (12). Briefly, the entire small intestine was removed and flushed with Ca^{2+} /Mg $^{2+}$ -free Hanks' balanced salt solution. Peyer's patches were dissected away, and the intestine was cut longitudinally into ≈ 10 -mm pieces. These pieces were incubated twice for 15 min at 37°C in phosphate-buffered saline/1 mM EDTA/1 mM dithiothreitol. Cells were collected from the supernatants

after each wash, filtered through nylon mesh, and washed once in RPMI 1640 medium. The pooled cells were then passed over a 40–80% discontinuous Percoll gradient. Cells at the interface were collected, washed several times, and kept on ice until use. The approximate yield was $1\text{--}2 \times 10^7$ cells per mouse. Single-cell suspensions of Peyer's patch lymphocytes were prepared from pooled, dissected Peyer's patches that were dissociated with glass microscope slides. Cells were maintained on ice in RPMI 1640 medium/10% fetal bovine serum until use.

Antibodies. Two mouse monoclonal antibodies (mAbs), TL.m4 and TL.m9, specific for the TLA expressed on ASL.1 thymic leukemia cells have been described (13) and were provided by E. Boyse and K. Kimura (Memorial Sloan-Kettering, New York). The TLA-specific rat mAb, HD168 (14) was from E. Stockert and L. Old (Memorial Sloan-Kettering). Mouse mAb 695 H1-7-3, which reacts with the Qa-2 determinant (15), was from D. Sachs (National Institutes of Health). Hamster mAb 145-2C11 is directed against the ϵ subunit of CD3 complex (16) and was from J. Bluestone (University of Chicago). Hamster mAb H57-597 recognizes mouse $\alpha\beta$ TCR (17) and was from R. Kubo (University of Colorado, Denver). mAb G8.8 reacts with intestinal epithelial cells and was from A. Farr (University of Washington, Seattle). Where indicated, the antibodies were biotinylated or directly coupled to fluorescein isothiocyanate (FITC) by using standard techniques (18). FITC-coupled anti-Lyt-2 was purchased from Becton Dickinson, and FITC-coupled anti-Thy-1 was purchased from Caltag Laboratories (San Francisco). Anti- $\gamma\delta$ TCR mAb GL3 was purchased from Pharmingen (San Diego).

Flow Cytometry Analysis. Cells were treated with the above mAb for 30 min at 4°C in phosphate-buffered saline, pH 7.4/2% bovine serum albumin/0.1% sodium azide and washed several times in the same buffer. When the mAb was not directly FITC-coupled, the cells were treated with an appropriate second antibody or streptavidin-phycoerythrin for 30 min at 4°C. Cells were then washed two times in staining buffer and resuspended in phosphate-buffered saline/1% paraformaldehyde. The samples were run on a FACScan flow cytometer (Becton Dickinson immunocytometry systems) equipped with a 15-mW 488-nm air-cooled argon-ion laser. The flow cytometer was equipped with Autocomp software (Becton Dickinson) that optimizes FSC (forward light scatter) and SSC [side (right angle) light scatter] settings to distinguish populations of lymphocytes from monocytes and granulocytes based on their size and granularity, respectively, and automatically adjusts the fluorescence photomultiplier tube settings and compensation. Five thousand events were acquired by using FACScan research (Becton Dickinson) software. The green fluorescence (FITC) channel uses a 530 ± 30 nm band-pass filter in front of the photomultiplier tube. The orange fluorescence (phycoerythrin) uses a 585 ± 42 nm band-pass filter. Single- and multi-parameter analysis using single histograms and contour plots and corresponding statistics were used. Overlays of histograms were created in FACScan research software.

Immunohistochemistry. Fresh tissues were snap-frozen in OCT embedding medium and stored at -80°C until use. Five-micrometer sections were cut on a microtome, fixed for 10 min in acetone, and stained in phosphate-buffered saline by using biotinylated mAb followed by avidin-horseradish peroxidase conjugates, as described (19). The sections were counterstained with Mayer's hematoxylin. Adjacent sections were stained with avidin-horseradish peroxidase conjugates alone to ensure specificity of the staining pattern.

Polymerase Chain Reaction (PCR) Analysis. RNA was prepared from tissues as described (20), and oligo(dT)-primed single-stranded cDNA was synthesized from 100 μg of total

RNA. Before cDNA synthesis, all RNA preparations were treated with RNase-free DNase (Stratagene) to eliminate contaminating genomic DNA. PCR amplification was conducted in a total volume of 50 μl using *Thermus aquaticus* (Taq) I polymerase (Cetus) in a thermal cycling machine (Ericomp, San Diego). Sequences of the *T18^d*-specific primers are as follows: (exon 2) 5'-ATAAGCTAAGTGC-CATGGGTGG-3' and (exon 3) 5'-TTATGAGCAGCATG-GCTATGATGGCC-3'. Sequences of the primers for the other *Tla* region genes are described elsewhere (P.E., K.B., R.H. and M.K., unpublished work). In this paper, we use the nomenclature for *Tla* genes recently proposed by Klein *et al.* (21). After 25 cycles, 10 μl of the PCR reaction was electrophoresed on 2% agarose gel, transferred to a nylon membrane (Oncor, Gaithersburg, MD), and hybridized with probes specific for the indicated *Tla* region genes (ref. 22, and P.E., K.B., R.H. and M.K., unpublished work). The membranes were washed at high stringency (65°C and $0.1 \times \text{SSC}$; $1 \times \text{SSC}$ is 0.15 M sodium chloride/0.015 M sodium citrate, pH 7.) and exposed for autoradiography.

RESULTS

Specificity of TLA mAbs. Results of previous serological studies have led to the designation of several *Tla* haplotypes based on reactivity of both polyclonal antibodies and mAbs with thymocytes from a variety of inbred mouse strains (13). We used the TLA-specific mAbs TL.m9 and TL.m4, both products of immunization of ($B6 \times A.Tla^b$)F₁ mice with the *Tla^a*-strain leukemia ASL1 (13). As summarized in Table 1, when cell suspensions were analyzed by flow cytometry, mAb TL.m9 was specific for thymocytes from *Tla^a* haplotype mice (e.g., B10.BR), mAb TL.m4 reacted with both *Tla^a* and *Tla^d* thymocytes (e.g., B10.BR and BALB/c), and neither antibody reacted with *Tla^b* thymocytes (e.g., C57BL/6) (data not shown). This pattern is consistent with that obtained with the same mAb by using other techniques, such as complement-mediated lysis (13). By using fibroblasts transfected with individual *Tla* genes cloned from BALB/c DNA, it has been shown that fibroblasts expressing *T18^d* but not other *Tla* region genes react with mAb TL.m4 (23). Rat mAb HD168 also reacted with thymocytes from both *Tla^a* and *Tla^d* haplotypes (23, 24) and stains transfectants expressing *T18^d* (R.H., K.B., H.C., and M.K., unpublished work).

Staining of Preparations of Thymocytes and IEL with TLA mAbs. Our preparations of murine thymocytes are a relatively homogenous population of small, nongranular cells when analyzed by flow cytometry by using the parameters of forward and right-angle light scatter. As expected, >90% of thymocytes from 7- to 10-week-old B10.BR mice stained brightly with the TLA-specific mAb TL.m9 (Fig. 1A). In marked contrast, our preparations of IEL are a heterogeneous mixture of cells containing both T lymphocytes and epithelial cells. These cell populations can be clearly distinguished and separated by gating on the basis of forward and right-angle light scatter. Based upon staining with a variety of antibodies and gating on different populations of cells, these preparations can be clearly divided into two distinct types of cells: (i) small nongranular cells that are >85% CD3⁺, ≈70% CD8⁺CD4[−], 5–10% CD8[−]CD4⁺, and 5–10% CD8⁺CD4⁺ (data not shown) and are, therefore, predominantly T lymphocytes and (ii) large granular cells that are <5% CD3⁺ and

Table 1. Strain specificity of anti-TLA mAbs

mAb	B10.BR (<i>Tla^a</i>)	C57BL/6 (<i>Tla^b</i>)	BALB/c (<i>Tla^d</i>)
TL.m4	++	−	+
TL.m9	++	−	−
HD168	++	−	+

Strain specificity is based on staining of thymocytes as described.

<5% CD8⁺ and that stain brightly with mAb G8.8, known to react with intestinal epithelial cells (data not shown, and A. Farr, personal communication); this latter population is composed mostly of intestinal epithelial cells.

Staining of an IEL preparation from B10.BR mice with mAb TL.m9 and gating on the different cell populations is shown in Fig. 1. In contrast to cells of Peyer's patches that did not stain appreciably for TLA (Fig. 1B), ~50% of the lymphocytes (IEL) in the preparation were TLA⁺ (Fig. 1C). Surprisingly most large, granular epithelial cells were also strongly TLA⁺ (Fig. 1D). As shown by Fig. 1, although reproducibly above background, the amount of TLA expressed on IEL was ~10-fold less than that seen on thymocytes. Because background staining was so much higher on epithelial cells than on lymphocytes, comparing relative amounts between these two cell types by using this technique is difficult. These results were confirmed by use of mAbs TL.m4 and HD168 on similar IEL preparations from both B10.BR and BALB/c mice (data not shown). Intestinal cells from C57BL/6 mice did not react with either mAb TL.m4 or mAb TL.m9. Note that the haplotype specificity of mAbs TL.m4 and TL.m9 in IEL preparations precisely followed what we and others have previously described for thymocytes (Table 1).

Greater than 90% of the lymphocytes in the IEL preparations stained brightly with several anti-Qa2 mAbs, whereas the epithelial cells were completely negative (data not shown).

Phenotypic Analysis of TLA⁺ IEL. Two-color flow-cytometric analysis with antibodies to a variety of T-lymphocyte surface markers was done to further analyze the population of IEL that express TLA. In general, we find that IEL contain roughly equal numbers of $\alpha\beta$ and $\gamma\delta$ T cells. Fig. 2 shows that TLA was expressed on most IEL expressing $\alpha\beta$ TCR but was found only on a small fraction of cells expressing $\gamma\delta$ TCR. Our IEL preparations contain 40–65% Thy-1⁺ cells. Antibodies to Thy-1 can stimulate T-cell proliferation (25), and Thy-1 is a reported marker of IEL activation (26). Most Thy-1⁺ cells in our preparations were TLA⁺, although there was a small population of Thy-1⁺ TLA⁺ cells (Fig. 2).

Immunohistochemistry on Intestinal Sections. To further clarify the location of the cells in the intestinal tract that express TLA, we examined its expression *in situ* by staining fixed tissue sections with the TLA-specific mAb. Fig. 3B shows that all luminal epithelial cells were positive for

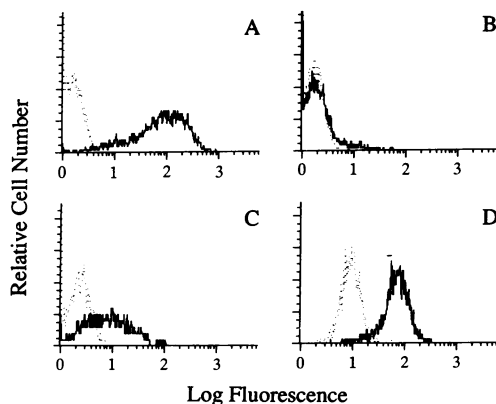


FIG. 1. Analysis of TLA expression in thymocytes and in IEL preparations. Cells from B10.BR mice were prepared as described and were stained with biotinylated mAb TL.m9 and then by streptavidin-phycoerythrin. For IEL preparations, cells were "gated" as lymphocytes or epithelial cells as described. Staining with mAb TL.m9 is shown by the solid line, and staining by streptavidin-phycoerythrin alone is shown by the dotted line. (A) Thymocytes. (B) Peyer's patch lymphocytes. (C) IEL. (D) Intestinal epithelial cells. Logarithms are to the base ten.

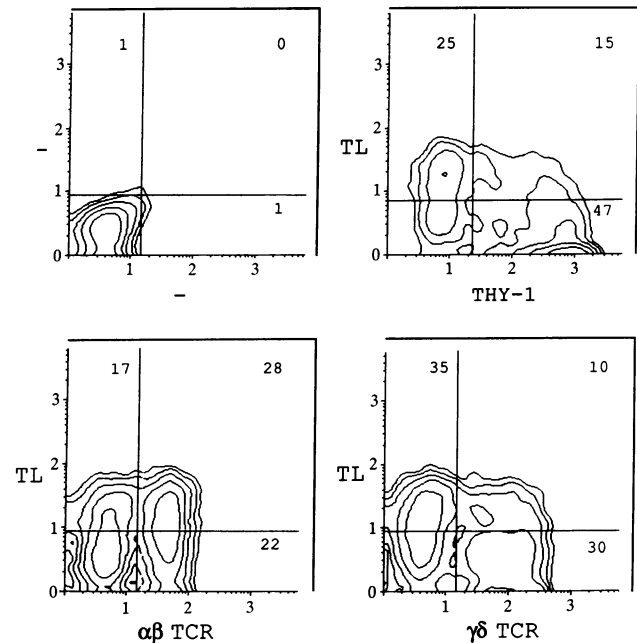


FIG. 2. Phenotypic analysis of TLA⁺ IEL. IEL were prepared as described from B10.BR mice, and 10⁶ cells were stained. The lymphocyte population was gated, and 5000 events were analyzed on a FACScan flow cytometer. (Upper Left) Profile seen with streptavidin-phycoerythrin alone. Incubation first with biotinylated TL.m9 and then with streptavidin-phycoerythrin and FITC-conjugated mAb Thy-1.2 (anti-Thy-1) (Upper Right), FITC-conjugated mAb H57-597 (anti- $\alpha\beta$ TCR) (Lower Left), or FITC-conjugated mAb GL3 (anti- $\gamma\delta$ TCR) (Lower Right). Scale logarithms (to base 10) indicate percent of cells in each quadrant.

surface expression of TLA. Moreover, staining density appeared more concentrated along the basal aspect of the epithelial layer. Both crypt cells and tip cells stained brightly, whereas the glandular epithelium, the lamina propria, and Peyer's patches did not stain. This contrasted markedly with the staining from the anti- $\alpha\beta$ TCR antibody; this antibody stained only T lymphocytes in the lamina propria, epithelia, and T-cell regions of Peyer's patches but did not stain the epithelial cells (Fig. 3A). The haplotype specificity of these antibodies in tissue sections also followed that described for thymocytes (Table 1) and IEL preparations. A similar pattern of cell-surface staining of the luminal epithelium was seen throughout the small intestine. TLA expression was not seen when sections of other epithelia reported to contain T cells expressing $\gamma\delta$ TCR (11), including tongue, esophagus, stomach, and female reproductive tract, were examined by this technique (data not shown).

To examine the role, if any, of lymphocytes in modulating expression of TLA by the epithelial cells, we stained intestinal tissue derived from *scid/scid* mice. The *scid/scid* mice did not contain any lymphocytes expressing $\alpha\beta$ TCR in the lamina propria or within the intestinal epithelium (data not shown). However, the staining pattern with the TLA mAb in *scid/scid* mice was identical to that seen in normal mice (Fig. 3C). This result indicates that TLA expression in intestinal epithelial cells is independent of the presence of mature functional T lymphocytes within the epithelium or lamina propria.

PCR Analysis of TLA Gene Expression. To confirm these results at the transcriptional level we performed PCR on cDNA from a variety of mouse tissues. Using primers specific for individual *Tla* genes, we were also able to investigate whether other *Tla* genes that encode products not recognized by available antibodies are transcribed in our IEL preparations. Because *Tla* genes appear inefficiently spliced

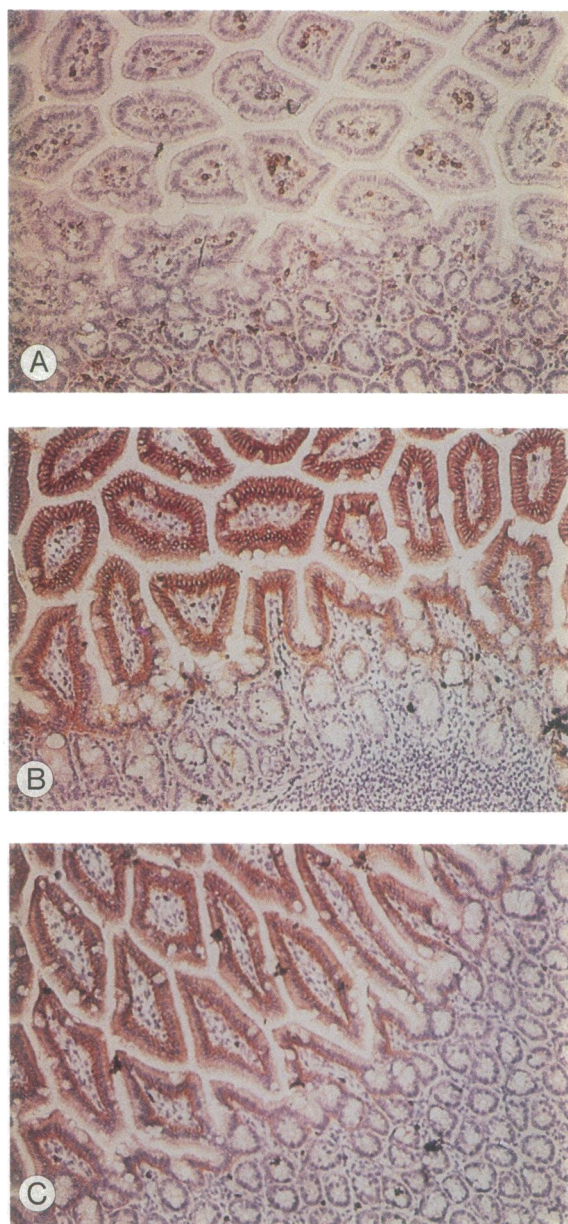


FIG. 3. Immunohistochemical analysis of small intestine sections. Sections ($5\ \mu\text{m}$) were prepared from the intestine of BALB/c and BALB/c *scid/scid* mice as described. The H_2O_2 -induced, peroxidase-dependent conversion of the colorimetric substrate results in the red color indicating the serological determinant recognized by the antibody. Mayer's hematoxylin counterstain results in the purple appearance of the cell nuclei. (A) Biotinylated mAb H57-597 staining of BALB/c mouse section. (B) Biotinylated mAb TL.m4 staining of BALB/c mouse section. (C) Biotinylated mAb TL.m4 staining of BALB/c *scid/scid* mouse section. ($\times 100$.)

in some tissues (27), it was of interest that both spliced and unspliced RNA were detected by using this technique. An example of this type of analysis with primers derived from exons 2 and 3 of *T18^d*, which are specific for this gene, is shown in Fig. 4. Both spliced and unspliced *T18^d* mRNA is seen in thymus (lane 1). Moreover, both unspliced and spliced *T18^d* mRNA was also seen in IEL preparations (lane 6). As the IEL preparations contain both lymphocytes and epithelial cells, this technique does not determine which cell type gives rise to the *T18^d* transcript. Nevertheless, this result is consistent with the staining pattern we observed in preparations of thymocytes and IEL with the TLA mAbs that appear to recognize the product of *T18^d*. No spliced *T18^d* gene transcripts were seen in adult liver, brain, heart, or mesen-

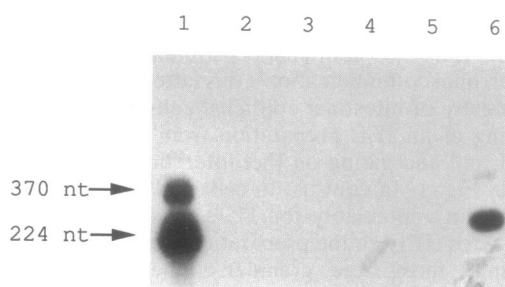


FIG. 4. PCR analysis of *T18^d* gene expression in IEL preparations. Ten-microliter aliquots of PCR reactions with primers specific for *T18^d* were electrophoresed on 2% agarose gel and transferred to a nylon membrane, as described. The membrane was then hybridized with *T3^d/T18^d*-specific DNA probe pTla 4, washed stringently, and exposed for autoradiography. Lanes: 1, thymus; 2, liver; 3, heart; 4, brain; 5, mesenteric lymph node; and 6, IEL preparation. Exposure time was 18 hr. nt, Nucleotides.

teric lymph nodes or several other tissues tested (data not shown).

We have performed similar analyses with primers specific for several other genes in the *Tla* gene region. We detected spliced mRNA in IEL preparations for both the *T10^d/22^d* gene pair (the members of which cannot be distinguished by PCR) and the *M2* gene (encoded in the *Hmt* region of the MHC) (28). In contrast, no unspliced or spliced mRNA in IEL is seen by using primers specific for the *T1^d*, *T2^d*, *T16^d*, *T17^d*, or *T24^d* genes (data not shown).

DISCUSSION

In this study we show that both intestinal epithelial cells and a significant fraction of IEL express the TLA on their surface. These observations were based on staining with three different anti-TLA mAbs in both single-cell suspensions and by *in situ* histochemical analysis. Reactivity of these antibodies in IEL preparations and intestinal sections followed the strain-distribution pattern we and others have seen in thymocytes. This result argues strongly that this staining pattern cannot be simply a cross-reactivity with another cell-surface antigen, unless it were closely linked genetically and, therefore, followed the identical strain distribution as TLA. Moreover, we confirmed by PCR analysis that spliced *T18^d* transcripts are present in the intestine and in the thymus, although absent from many other tissues tested.

The expression of TLA in a population of mature lymphocytes in the intestinal epithelium is somewhat surprising in the context of the lack of TLA expression in other peripheral sites (e.g., lymph nodes and Peyer's patches). TLA expression has been reported to be induced in splenic T cells *in vitro* after treatment with Con A (4). These data suggest that TLA may be a marker of, or play some role in, T-cell activation. In our study, most of the IEL expressing $\alpha\beta$ TCR and a lesser proportion of the IEL expressing $\gamma\delta$ TCR express TLA. Lefrancois and Goodman (29) have reported that a population of IEL are constitutively lytic. This lytic activity was hypothesized to result from *in vivo* activation and was subsequently shown to be independent of the type of TCR expressed (i.e., $\alpha\beta$ or $\gamma\delta$) (30). The activated IEL in these studies were believed to be Thy-1^+ (26). If TLA were, indeed, a marker of IEL activation, the presence of TLA on a significant fraction of IEL would be consistent with these observations. However, most of the TLA^+ IEL in our preparations are Thy-1^- . An alternative explanation for the TLA expression profile in IEL is that the TLA^+ and TLA^- lymphocytes are derived from distinct lineages of T lymphocytes (i.e., thymic or extrathymic).

The presence of the TLA on the surface of epithelial cells in the mouse small intestine extends the known profile of expression of this molecule. Previously, TLA has been detected only in cells of the hematopoietic lineage, including T cells and Langerhans cells of the skin (31). Transcripts of some other *Tla* region genes that do not encode serologically detectable proteins have been shown to have a wider tissue distribution at the RNA level (32–34). However, in these instances, the cell type expressing the *Tla*-encoded gene product on its surface has not been identified.

The presence of TLA on the surface of the intestinal epithelium is especially interesting in view of recent reports by several groups describing $\gamma\delta$ T-cell lines or T-cell hybridomas alloreactive to or restricted to products of the *Tla* region of the MHC (6–8). IEL are predominantly CD4[−]CD8⁺ and, therefore, are likely to interact with class I or class I-like molecules. It is tempting to speculate, as others have (9, 10), that the products of *Tla* genes may be involved in presenting peptide antigens to T cells bearing $\gamma\delta$ TCR. *Tla* gene products could also have a role in antigen presentation to T cells bearing $\alpha\beta$ TCR. It has been recently reported that *Tla* molecules can be involved in the negative selection of $\gamma\delta$ TCR expression in transgenic mice (35). As $\gamma\delta$ IEL are seen in *nu/nu* mice and may, therefore, mature extrathymically (36), TLA could also be involved in the education of $\gamma\delta$ T cells in the intestinal epithelium.

Mayer and Schlein (37) have shown that intestinal epithelial cells can function as antigen-presenting cells to splenic or peripheral blood-derived T cells *in vitro*. It is, therefore, likely that intestinal epithelial cells are involved in the presentation of antigen to lymphocytes present within the epithelial barrier *in vivo*. Although the present data do not provide direct support for the hypothesis that *Tla* gene products can present antigen to T cells that express $\gamma\delta$ TCR, the demonstration of TLA on putative antigen-presenting cells in an anatomical site enriched for such cells makes the hypothesis tenable. These data extend the possible functional role for TLA beyond the proposed role in T-cell development and/or proliferation.

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